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TITLE: Endocannabinoids as a Target for the Treatment of Traumatic Brain Injury

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14. ABSTRACT
The results from our ongoing studies during this funding period have provided evidence that EC enzyme degradation inhibitors are effecting at reducing astrocyte and microglial activation at 24hrs post-TBI. In addition, perfusion fixed brains with one dose of the EC enzyme degradation inhibitors at 7 and 30 day post-TBI will undergo immunostaining to evaluate the injury lesion size and detection of activation of astrocytes and microglia, as well as neuronal cell death and density. Finally, oxidative stress related mRNA and protein expression will be measured in samples obtained 72 hrs post-TBI following two doses of the EC enzyme degradation inhibitors.

15. SUBJECT TERMS

Lateral fluid percussion, traumatic brain injury, blood brain barrier, neuroinflammation, neurological dysfunction, endocannabinoids, microglia and astrocyte activation.

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INTRODUCTION:

Long term effects of traumatic brain injury (TBI), including neuroendocrine dysregulation and neurobehavioral recovery may be ameliorated by intervention aimed at reducing short term neuroinflammation, oxidative stress, and altered neuroendocrine and behavioral functions. Our working hypothesis is that maintaining levels of the endocannabinoids (EC), 2-arachidonoyl glycerol (2-AG) and N-arachidonoyl ethanolamine (AEA) should reduce neuroinflammatory changes following TBI. During this funding year, selective pharmacologic inhibitors have been used to decrease the degradation of 2-AG and/or AEA by a single dose of the inhibitors at 24 and 72 hrs, and 7 and 30 days post-Injury. In addition, studies during this funding year were focused on extending the duration of drug treatment to establish the optimal length of intervention. This is accomplished by administrating dose of the selective EC enzyme degradation inhibitors 30 min post-TBI, as well as an additional dose at 24 hrs post-TBI. The goal of these studies is to determine if this pharmacological regimen will reduce TBI-induced neuroinflammation, blood brain barrier (BBB) permeability and neurobehavioral dysfunction. Ongoing studies in the upcoming year (no cost extension requested) will continue to examine the extent to which this intervention modulates cytokine release, neutrophil influx, blood brain barrier permeability, and neurological and neurobehavioral severity impairments following TBI.

BODY:

Progress Report NCE Annual Funding Period:

Studies to date have been directed towards the completion of Milestones 1, 2, and 3 as defined in the Statement of Work. The goal of milestone 1 is to describe the impact of EC degradation inhibition on neutrophil influx, pro-inflammatory cytokine expression, oxidative injury, edema, and blood barrier permeability. Additionally, histological assessment of the protective effects of EC following brain injury will be demonstrated. The goal of milestone 2 is to examine the effectiveness of decreasing EC degradation in maintaining neuroendocrine integrity following TBI. The goal of milestone 3 is to examine the efficacy of elevated EC levels to provide neuroprotection and improve neurobehavioral outcome as reflected in motor and cognitive function. Current efforts are focused primarily on Tasks 1 and 3.

Task 1: Determine the effectiveness of specific inhibitors of endocannabinoid degradation in reducing neutrophil influx, pro-inflammatory cytokine expression, oxidative injury, edema, and blood barrier permeability.

a. ***Inflammation & oxidative stress (2h, 4h, 24h, 72h post-TBI)*** . Brain tissue (area of injury, prenumbral region, contralateral region, frontal cortex) content of cytokines and chemokines, oxidative stress (lipid peroxidation and catalase activity). Inflammatory cell infiltration examined by immunohistochemistry.
b. Brain edema (4h, 24h, 72h post-TBI). Wet/dry ratio determined.
c. Blood brain barrier permeability analyzed by dye tracer extravasation (24h & 72h post-TBI).
d. ***Cell injury by histological analysis (7d & 30d post TBI).***

e. Endo-Cannabinoid Levels measure in extracted brain tissue lipids

Progress:

Efforts under Task 1 have been primarily focused towards Task 1a (Inflammation and oxidative stress), and Task 1d (Cell injury by histological analysis). For Task 1a, previously collected tissues at 24hrs post-TBI have been analyzed for protein expression of cannabinoid receptors 1 & 2 (CB1, CB2) and iNOS. In addition tissues were analyzed for both mRNA and protein expression of COX2 and NOX2 at 24 and 72hrs post-TBI

In addition, progress has been made towards Task 1d, cell Injury by histological analysis from sham (un-injured) and TBI animals with a single dose of EC degradation inhibitors (JZL184; 16 mg/kg and URB597 0.3 mg/kg, IP) administered 30 min post-TBI. Brains were perfused for one set of animals at 24hrs post-TBI and were sliced to evaluate the level of activation of microglia and astrocytes, sections were stained using specific antibodies (ED-1 - microglia and GFAP – astrocytes).

Summary of Findings:

Task 1a

Cannabinoid Receptor Protein Expression
Following Traumatic Brain Injury

TBI initiates a neuroinflammatory cascade characterized by an increased production of oxidative radicals which can potentially increase the secondary damage post-TBI and lead to neurodegeneration. We have previously demonstrated that treatments to the endocannabinoid system by inhibiting the degradation enzymes of 2-arachidonoyl glycerol (2-AG) and N-arachidonoyl-ethanolamine (AEA) reduce the neuroinflammatory response at 24 hrs post-TBI. In the first quarter, tissue samples were collected at 24hrs post-TBI and analyzed for cannabinoid receptors 1 and 2 (CB1 and CB2) protein expression using Western blot analysis (Fig. 1A-B). Current data demonstrates no significant differences between Sham and TBI/Vehicle treated animals. Furthermore, drug interventions (JZL184 16mg/kg, IP, 30min's post-TBI) and (URB597 0.3mg/kg, IP, 30 min's post-TBI) did not effectively alter the protein expression of CB1 and CB2 at 24 hrs post-TBI.

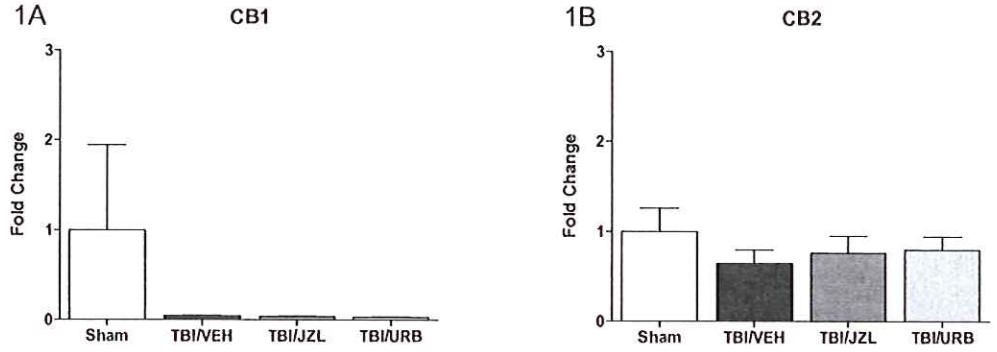


Fig. 1A-B: Cannabinoid receptor protein expression measured in the ipsilateral brain region 24hrs post- TBI. Inhibition of 2-AG and AEA degradation by the use of the selective inhibitors (JZL184; 16 mg/kg and URB597 0.3 mg/kg, respectively, IP) administered 30 min post-TBI does not appear to alter the protein expression of CB1, and CB2. (n=4/group).

Oxidative Stress Related mRNA and Protein Expression Following Traumatic Brain Injury

In the second quarter of this funding year, tissue samples collected at 24 and 72hrs post-TBI were analyzed for COX2 and inducible nitric oxide synthase (iNOS) mRNA and protein expression (Fig. 2A-D). This data demonstrates no significant differences in mRNA or protein expression of COX2 and iNOS at 24 and 72 hrs post-TBI between Sham and TBI/Vehicle treated animals. Furthermore, drug interventions (JZL184 16mg/kg, IP, 30min's post-TBI) and (URB597 0.3mg/kg, IP, 30 min's post-TBI) did not significantly alter the mRNA or protein expression of COX2 and iNOS at 24 hrs post-TBI. In addition, tissue samples at 24 hrs post-TBI were analyzed for NOX2 mRNA and protein expression (Fig. 3A-B). These results show no significant differences in NOX2 mRNA or protein expression at 24hrs post-TBI between SHAM and all TBI groups.

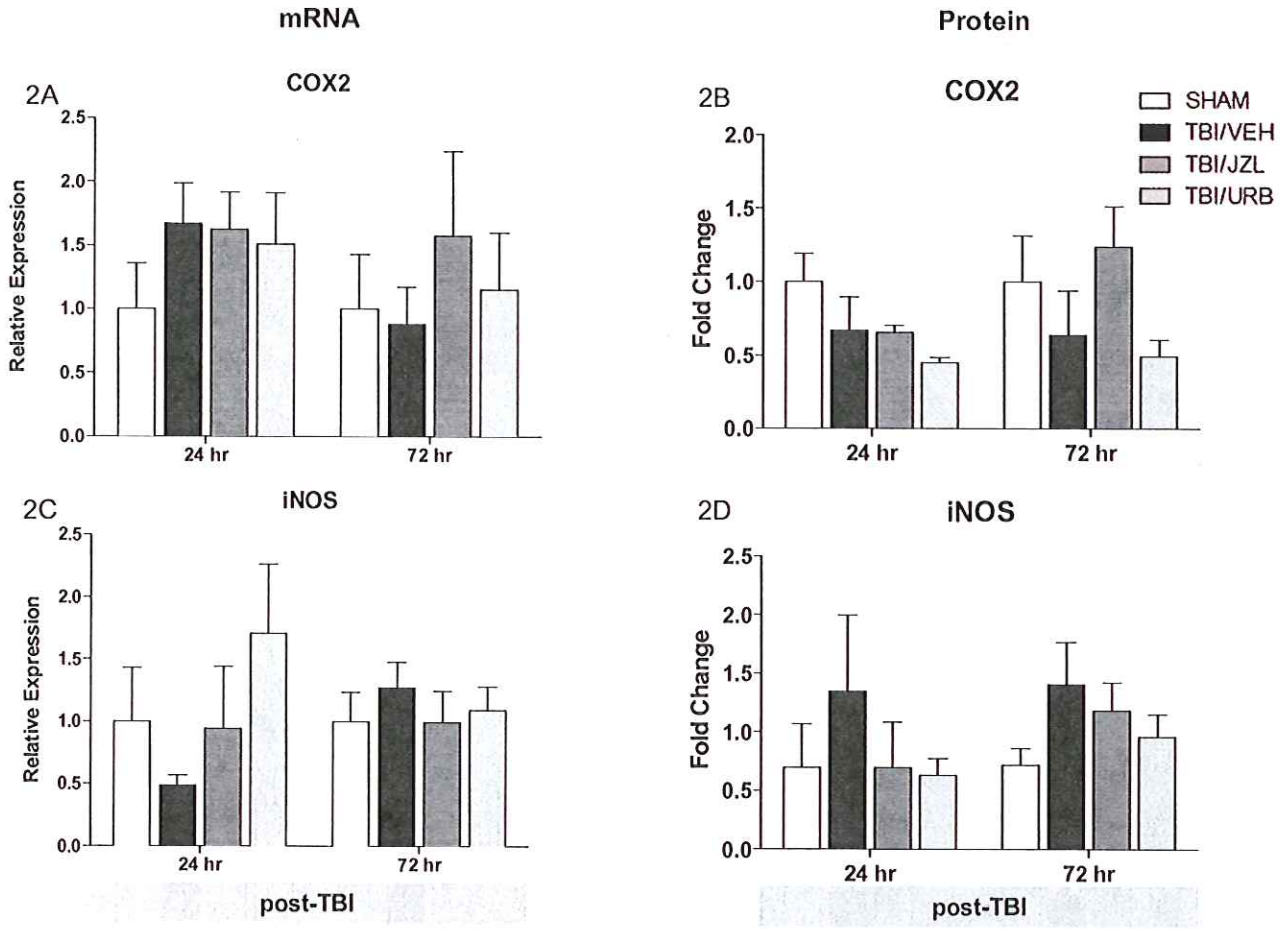


Fig 2A-D: Protein and mRNA expression of COX2 and iNO2 measured in the ipsilateral brain region 24 and 72 hrs post- TBI. Inhibition of 2-AG and AEA degradation by the use of the selective inhibitors (JZL184; 16 mg/kg and URB597 0.3 mg/kg, respectively, IP) administered 30 min post-TBI does not appear to alter the protein (A&C), expressed as relative expression normalized to β-actin or mRNA (B&D), expressed as fold change over SHAM of COX2 or iNOS. (n=4-8/group).

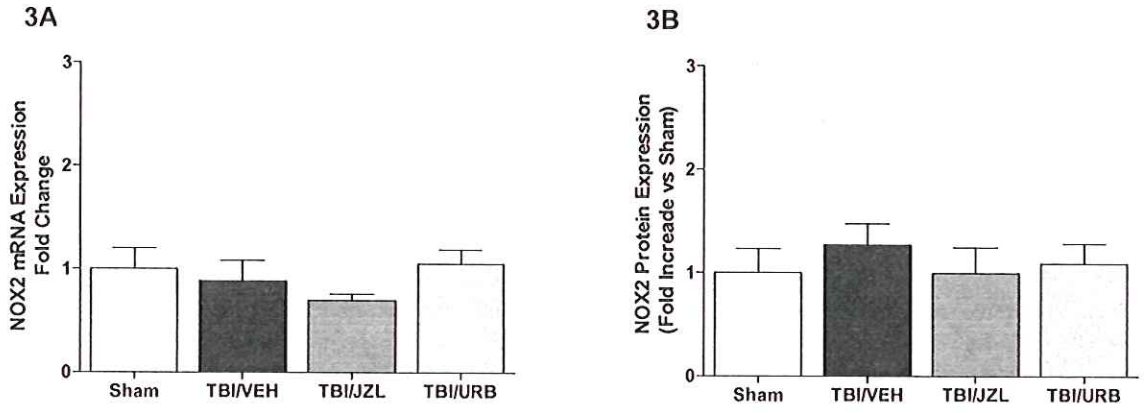
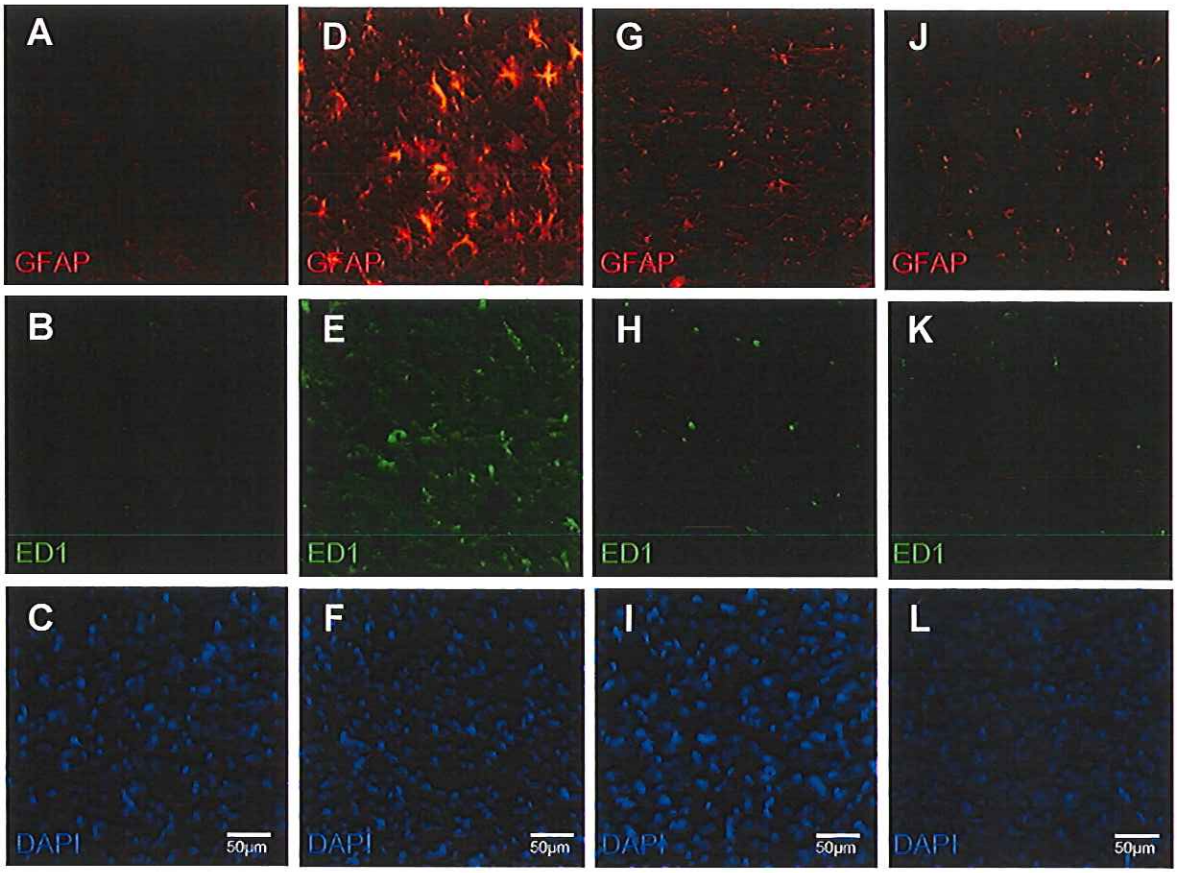


Fig 3A-B: mRNA and protein expression measured in the ipsilateral brain region 24 and 72 hrs post- TBI. Inhibition of 2-AG and AEA degradation by the use of the selective inhibitors (JZL184; 16 mg/kg and URB597 0.3 mg/kg, respectively, IP) administered 30 min post-TBI does not appear to alter the mRNA (A), expressed as fold change over SHAM or protein (B), expressed as fold increase vs SHAM after normalization to β-actin expression of COX2 or iNOS. (n=4-8/group).

Astrocyte and Microglial Activation Following Traumatic Brain Injury

In addition to oxidative stress, activation of resident support cells, microglia and astrocytes, [post-injury can play a role in sustained neuroinflammation that increase secondary injury. In the second quarter of this funding year, perfusion fixed brains were sliced, mounted and stained with GFAP, a marker of astrocyte activation, and ED1, a marker of microglial activation. Analysis was completed on perfusion fixed brains 24 post-TBI. (Fig. 4A-N).



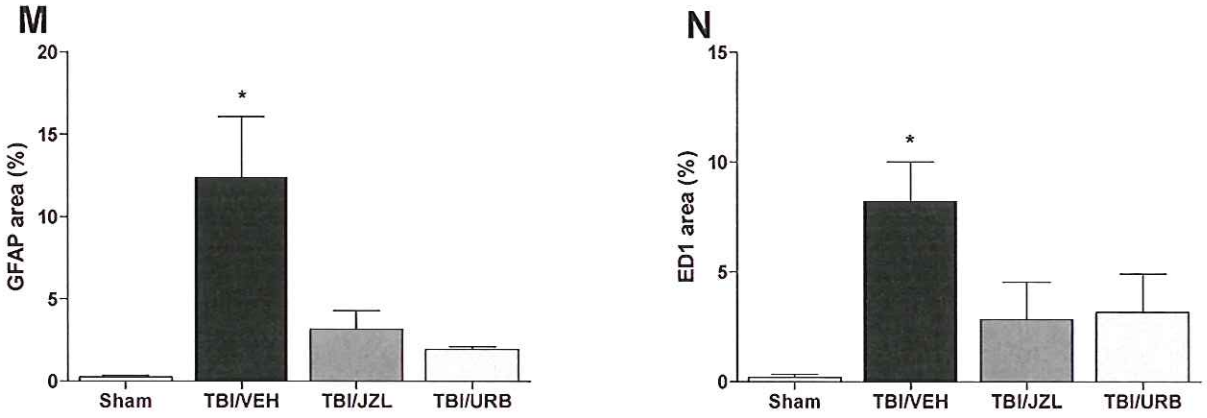


Fig 4A-N: Immunohistochemistry of perfusion fixed brains 24 hr post-TBI. Inhibition of 2-AG and AEA degradation by the use of the selective inhibitors (JZL184; 16 mg/kg and URB597 0.3 mg/kg, respectively, IP) administered 30 min post-TBI, significantly reduced astrocyte (GFAP) and microglial (ED1) activation at 24 hrs post-TBI. (n=4-8/group).

Recent efforts have been focused on collection and processing of perfusion fixed 7 and 30 day post-TBI tissue for neuronal cell injury and activation of astrocytes and microglia. We have now collected an n=6 for each treatment group (SHAM, TBI/VEH, TBI/JZL and TBI/URB) treated with a single dose of the endocannabinoid degradation inhibitors 30 min post-TBI.

Task 2: Determine the effectiveness of the selective increase in endogenous 2-AG and AEA levels in preventing neuroendocrine dysfunction following TBI.

a. Basal unstimulated neuroendocrine function
b. Autonomic and neuroendocrine response to cardiovascular challenge
c. Autonomic and neuroendocrine response to water deprivation test

Progress:

No progress to report.

Task 3: Determine the capacity of increased EC levels to protect neurobehavioral and cognitive function following TBI.

a. Severity of TBI determined by the righting reflex.
b. Sensory reflex examined by the forelimb and hindlimb reflex.
c. Somatomotor function examined by a beam balance task and beam-walking task.
d. Cognitive function tested by the radial-arm maze.

Progress:

Ongoing efforts have been directed towards the completion of Tasks 3a-d using a double dose of EC enzyme degradation inhibitors. These initial studies have focused on assessing the effects of a double dose of EC enzyme degradation inhibitors on neurological and neurobehavioral outcomes following TBI. Neurological (NSS) and Neurobehavioral (NBS) function is examined by a combination of somatomotor and cognitive assessments. Following TBI, each animal is re-assessed at 2, 24, 48, and 72hrs time points to determine the degree of neurological and neurobehavioral dysfunction. The selective EC enzyme degradation inhibitors (JZL184; 16 mg/kg and URB597 0.3 mg/kg, IP) are administered 30 minutes post-TBI followed by a second dose given 24 hrs later. Previous results have demonstrated that a single dose of JZL184 administered 30 minutes following injury is superior to URB597 in improving blood brain barrier integrity, neuroinflammation, neurological (NSS) and neurobehavioral (NBS) outcomes following TBI..

KEY RESEARCH ACCOMPLISHMENTS:

- EC enzyme degradation inhibitors, given at 30 min post-TBI, are effective at reducing neuroinflammation as indicated by reduced GFAP and ED-1 expression, makers of astrocyte and microglial activation, respectively at 24 hrs post-TBI.

REPORTABLE OUTCOMES:

Publications:

Book chapter published.

Katz PS, Edwards S, Molina PE. Cannabinoids. ***Neuroinflammation and Neurodegeneration.*** *Peterson and Torborek (eds.), Springer Science + Business Media, New York, NY, 2014.*

Manuscript published.

Katz PS, Sulzer J, Impastato R, Teng X, Rogers E, Molina PE. Endocannabinoid degradation inhibition improves neurobehavioral function, blood brain barrier integrity and neuroinflammation following mild traumatic brain injury. Journal of Neurotrauma, 2014 Aug 28 [Epub ahead of print]

Presentations:

Poster presentation at Experimental Biolog, April 2013.

Katz PS, Impastato R, Rogers E, Molina P. Inhibition of endocannabinoid degradation reduces neurological damage and blood brain barrier disruption following traumatic brain injury.

CONCLUSION:

The results from our ongoing studies during this funding period have provided evidence that EC enzyme degradation inhibitors are effecting at reducing astrocyte and microglial activation at 24hrs post-TBI. In addition, perfusion fixed brains with one dose of the EC enzyme degradation inhibitors at 7 and 30 day post-TBI will undergo immunostaining to evaluate the injury lesion size and detection of activation of astrocytes and microglia, as well as neuronal cell death and density. Finally, oxidative stress related mRNA and protein expression will be measured in samples obtained 72 hrs post-TBI following two doses of the EC enzyme degradation inhibitors.

REFERENCES: N/A

APPENDICES: N/A